

## THROMBOSIS ET DIATHESIS HAEMORRHAGICA

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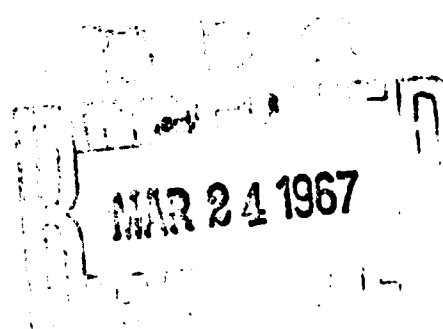
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**Effects of Staphylococcal Enterotoxin B  
on the Coagulation Mechanism and Leukocytic  
Response in Beagle Dogs – A Preliminary Study**

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Many clinical syndromes are associated with infections in which hemorrhage is a prominent feature. At the present time, however, there is only a paucity of information available concerning the effect of infectious agents and biological products on the coagulation of blood. The pathogenesis of the hemorrhage in most instances is poorly understood, and it is not known whether the damage resulting in bleeding is mediated by a direct action of the organism or toxin or indirectly by some unknown mechanism. Intimately associated, but equally as obscure, are the alterations in the vascular, cellular, and plasma factors responsible for hemostasis. It is well known that the delicate balance of these factors is maintained by several organs or organ systems and that minimal dysfunction is sufficient to alter one or a combination of the factors. Such an alteration may be manifested clinically by mild purpura, overt bleeding, profound shock associated with intravascular coagulation, or by thrombosis and its sequelae. It is obvious then that much exploration is needed in the area of infectious disease and blood coagulation and that the basic questions of pathogenesis, effective therapy and sound prophylaxis are, with few exceptions, outstanding.

The present study was designed to define some of the basic hemostatic alterations in dogs receiving a highly purified staphylococcal enterotoxin.

### **Materials and Methods**

Ten young adult purebred beagle dogs weighing 8.2–12.7 kg were given highly purified staphylococcal enterotoxin B<sup>(1)</sup> intravenously. Nine dogs received 100 µg/kg; one, 50 µg/kg. Eight ml of

The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed in this study.

blood were obtained from the external jugular vein 4 days before toxin administration and at varying time intervals after challenge. Nine parts of whole blood were anticoagulated with 1 part of 3.2% sodium citrate. The plasma was separated immediately. The whole blood clotting time was performed by a modification of the Lee-White method<sup>(2)</sup>. These tubes were maintained at room temperature and were observed at hourly intervals for evidence of clot retraction and at 72 hrs for evidence of lysis. Retraction was graded from 3+ to 0. The prothrombin time tests (PT) were performed by the method of Quick<sup>(3)</sup> and the partial thromboplastin time tests (PTT) by the method of Langdell, Wagner, and Brinkhous (4). Each sample was tested in triplicate.

Twenty-two normal purebred beagle dogs were used to establish the range and mean of the PT and PTT. The mean of the PT for the group was 9.3 sec; the mean of the PTT was 27.7 sec. The ranges are indicated by the shaded areas in Fig. 3 and 4. Ten dogs were randomly selected from this normal group for challenge with enterotoxin. The mean of the PT and PTT for each of the post-challenge bleeding periods was established.

## Results

All dogs became ill within 3 hours after toxin administration as evidenced by emesis and diarrhea. One dog died 13 hrs postchallenge; another, in 4 days.

*Whole blood clotting time (CT):* The prechallenge CT for the 10 dogs ranged from 5 min 54 sec to 10 min 33 sec; the mean was 8 min 20 sec. Fig. 1 illustrates the average clotting times for each bleeding period and depicts the prolongation immediately after challenge and the gradual prolongation to 22 min on the 28th day. The CT returned to the upper normal range by the 51st day.

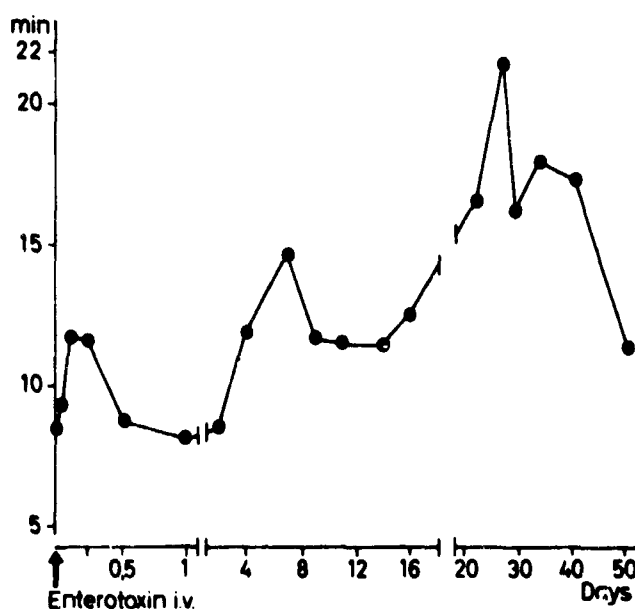


Fig. 1. Changes in whole blood clotting time

*Clot retraction (Fig. 2):* Clots retracted normally (3+) before challenge and at the 1-hour postchallenge sampling. Beginning at 3 hrs, however, there was

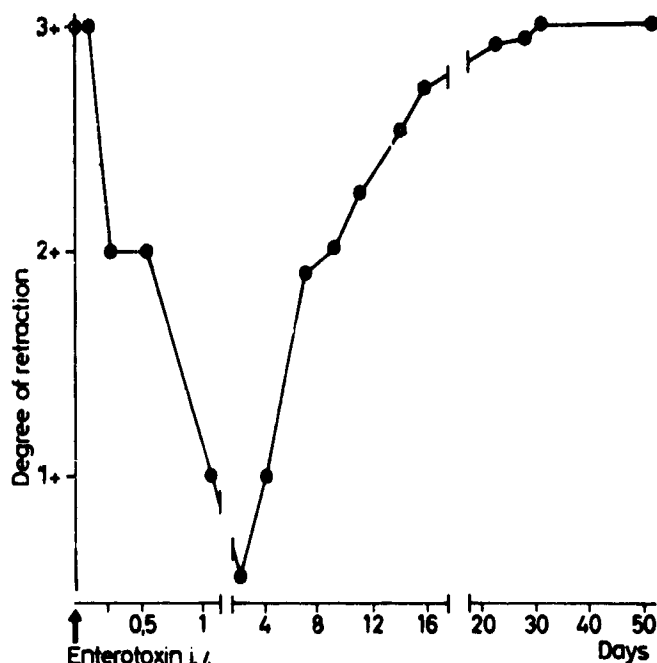


Fig. 2. Change in clot Retraction

a diminution of retraction (2+) which continued until the second postchallenge day when only a slight degree of retraction was demonstrable in 2 of the 9 samples. On the fourth postchallenge day, a barely perceptible clot (1+) was present in each tube, but retraction improved slowly during the following 18–20 days. Retraction returned to normal on the 23rd postchallenge day and remained normal during the 28 additional days of the study.

**Clot lysis:** No liquefaction of coagulated blood occurred during a 72 hr observation period. Once formed and after retraction, the clots did not change in size or consistency.

**Prothrombin time (PT):** Fig. 3 illustrates the prechallenge and postchallenge averages of the PT for each bleeding period and the prolongation at the 6 hr bleeding. Subsequently, the PT returned to or was slightly below the established normal range.

**Partial thromboplastin time (PTT):** The prechallenge mean and postchallenge variations in the PTT are presented in Fig. 4. Within 6 hrs after challenge a prolongation of the PTT occurred and persisted until the fourth day. Thereafter the values returned to the normal range. A shortening to slightly below the lower normal range was sporadically present after the 20th day.

**Leukocyte count:** The prechallenge leukocyte count ranged from 9,850 to 21,300/mm<sup>3</sup>; the mean was 16,844/mm<sup>3</sup>. Fig. 5 illustrates the leukopenia of 1,830/mm<sup>3</sup> at the 1-hour postchallenge sampling; the range was 950 to 3,400/mm<sup>3</sup>. The mean at 3 hrs was 1,890/mm<sup>3</sup>; the range was similar to the 1-hour value. Thereafter, a leukocytosis gradually developed and reached an

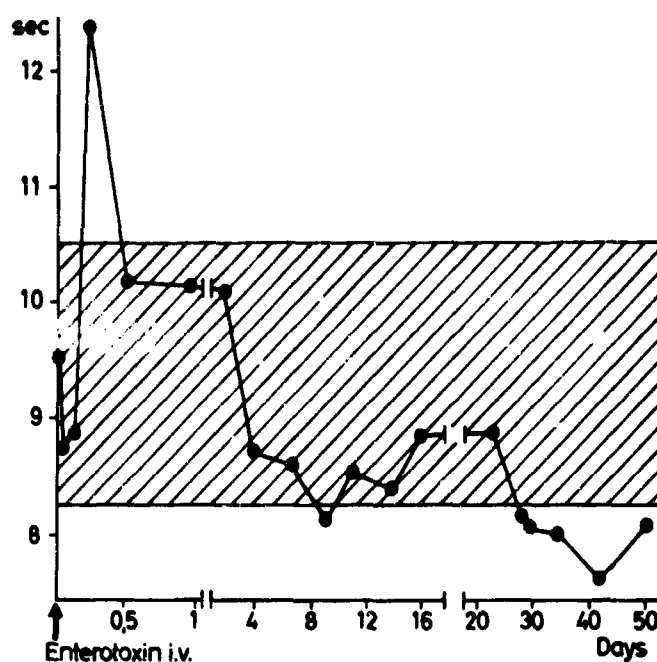


Fig. 3. Variation in prothrombin time

average of 19,100/mm<sup>3</sup> on the fourth day. Two additional elevations occurred on the 11th and 51st days.

*Differential leukocyte count (Fig. 6):* The mean of the prechallenge differential count was 69% neutrophils, 30% lymphocytes and 1% monocytes. One hour after challenge the neutrophils and lymphocytes were present in almost equal

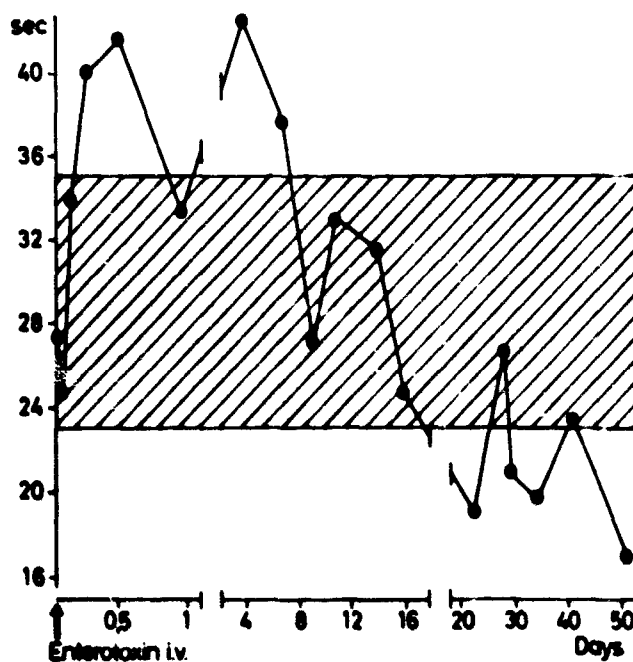


Fig. 4. Variation in partial thromboplastin time

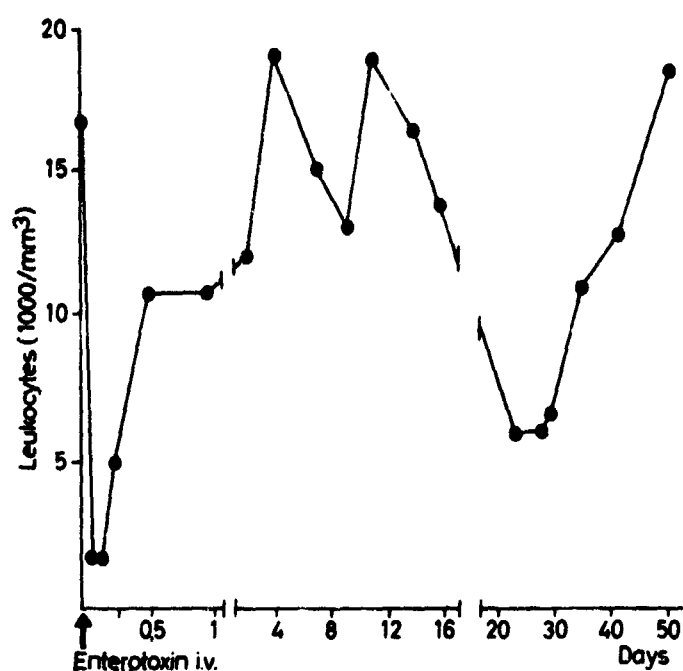


Fig. 5. Total leukocyte count

numbers: 52% and 46%, respectively. During the next two days, the neutrophils increased to 83% and the lymphocytes dropped to a low of 13%. On the 4th postchallenge day and continuing through the 16th day the ratio was within normal range. A reversal of the ratio occurred on the 23rd day with 41% neutrophils and 57% lymphocytes; the cells were present in equal numbers on the

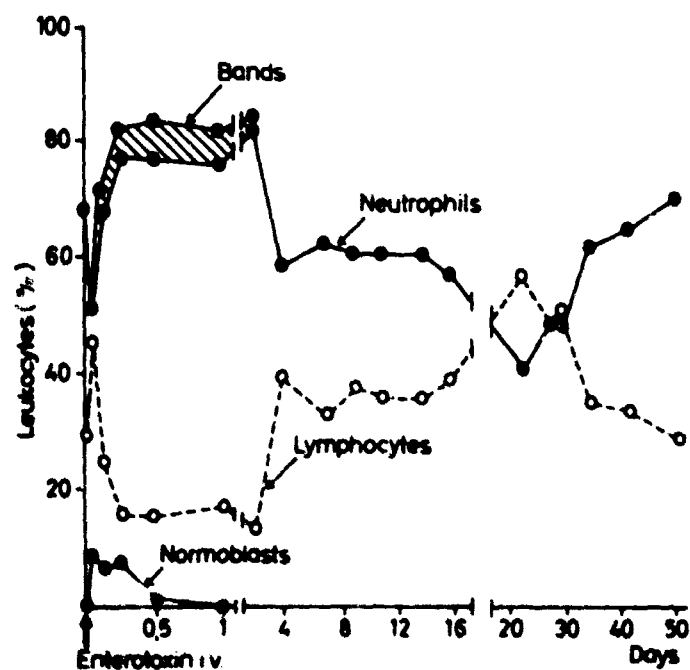


Fig. 6. Differential leukocyte count

28th and 30th days. Thereafter, the ratio returned to and was normal on the final count.

Three hours after challenge a mild "shift to the left" began and reached an average of 7.5% band forms at 12 hrs. The immature leukocytes had disappeared in the 48 hr sample.

No normoblasts were observed among the 100 leukocytes counted on the prechallenge sample. At one hour, however, an average of 9 normoblasts was present among 100 leukocytes (Fig. 6); each animal had at least 1, and one had 25. An average of 6 and 7 normoblasts were present at 3 and 6 hrs and decreased to 2 within 12 hrs. Thereafter, only an occasional nucleated erythrocyte was seen.

*Autopsy findings:* A complete autopsy was performed on the 2 animals that died. One died 13 hrs after challenge and had lost 1.5 kg body weight. The second died in about 4 days and lost 2.2 kg. Each animal had a severe enterocolitis with erosion and ulceration. Another prominent feature in each was the hemorrhagic character of the thymus, lymph nodes, spleen, and liver. This finding was most pronounced in the animal that died in 13 hrs. Sections of sternal marrow from each were normocellular. There was marked lymphadenopathy which was secondary to reticuloendothelial hyperplasia, germinal center necrosis, focal hemorrhage and leukocytic infiltration. Death in the first animal probably resulted from blood loss and dehydration. The second animal had a severe necrotizing pneumonia with numerous clumps of bacteria in the areas of necrosis. No significant changes were present in other organs. There was no histologic evidence of thrombosis or infarction.

### Discussion

Changes occurred in all of the parameters tested except for clot lysis and were most marked during the first four postchallenge days.

The immediate effect associated with the toxin was an abrupt decrease in the total white blood count to a leukopenic range ( $1,830/\text{mm}^3$  average). This occurred without exception in every animal and was the first response noted, including the clinical manifestations of illness. A similar decrease has been noted in rabbits receiving a relatively pure endotoxin (6). All animals were ill within 2 hrs and 15 min, however, as evidenced by vomiting. The pathogenesis of the leukopenia is unknown but is probably similar to that which occurs in overwhelming bacterial infections or septicemias.

At the present time several mechanisms have been proposed to explain the leukopenia of infections. Injection of bacterial proteins results in an immediate reduction of polymorphonuclear leukocytes to leukopenic ranges (7). Associated

with some bacterial infections is the production of a leukopenia-producing substance (8) which results in the trapping of white cells in the lungs or in the reticuloendothelial system. It is thought that this is the mechanism of leukopenia in typhoid infections (9). A toxic effect on the bone marrow resulting in diminished production of cells is an important consideration, but two findings in the present study indicated that the marrow was not affected by the toxin. One is the normal morphology of the marrow in those animals that died, and the second is the measurable observations of an actively functioning marrow in all animals during life. Another consideration too important to disregard is the rapidity with which the cells are depleted from the circulation. The leukopenia was present 1 hr after administration of the toxin and may have occurred earlier. A slight to moderate increase of white cells was present in the spleen and lymphoid tissue of the 2 animals that died. This may be contributory but is thought not to be marked enough to explain the leukopenia. Loss from the gastrointestinal tract may be more important since numerous viable and degenerate white cells were present in the lamina propria, especially of the colon and small intestine. Accumulation of white cells did not occur in the liver or lungs of the first animal that died. All facts strongly suggest that removal or destruction rather than impaired production is the mechanism by which the leukopenia developed.

The initial leukopenia was followed by a leukocytosis that reached an average of about  $20,000/\text{mm}^3$  in 4 days. The elevation persisted until 14 days postchallenge when the values began to return to normal. No evidence of clinical illness was noted on the 51st postchallenge day when the leukocyte count was again increased.

With the initial leukocytosis there was a mild "shift to the left" that continued for 2 days. Concomitant with this on the first day was the appearance of normoblasts in the blood. The findings of early forms of each series suggested that the marrow was active, capable of responding, and not impaired by the toxin.

The coagulation time increased 4 min beyond the mean of 8.2 min during the first 6 hrs postchallenge and returned to the baseline value in 12 hrs. This is in contrast to an initial shortening observed immediately following an intravenous injection of endotoxin (10). This increase paralleled the prolongation of the PT and PTT. Another prolongation to almost 15 min occurred around the 7th day but was not associated with changes in either the PT or PTT. Thereafter, the CT remained longer than 10 min and reached a maximum of almost 22 min on the 28th postchallenge day. None of the parameters tested were abnormal at this time, but this finding does suggest a deficiency of at least one of the plasma factors.



Severely impaired clot retraction was present from 1 to 4 days after challenge but gradually returned to normal about the 20th day. Platelets appeared to be present in adequate numbers in each of the blood smears. A quantitative decrease in platelets has been reported in rabbits after intravenous administration of staphylococcal enterotoxin (11), and *in vitro* studies indicate that platelets are destroyed or damaged by the toxin (12). A marked reduction in the number of circulating platelets has been observed in rabbit after endotoxin (6), and a decrease in retraction capacity of clots from rabbits given endotoxin has been demonstrated using thromboelastography (13). The latter finding was directly related to a decrease in platelet adenosine triphosphate. Exploration of the factors affecting retraction was not attempted in this study.

The immediate decrease in shortening of the PT and PTT was slight and may be explained by variations inherent in the test itself. The prolongation of each test beginning with the 6 hr sampling, however, is more than can be accounted for by variations in the technique alone.

The PT increased to 12.4 sec from a prechallenge mean of 9.5 sec after 6 hrs. The PT is usually prolonged with an absence or deficiency of factors V (proaccelerin), VII (SPCA), or X (Stuart). A reduction of the factors may be associated with hemorrhage, intravascular coagulation or any disorder affecting their synthesis or release by the liver. From the present studies, it can only be concluded that alterations are present and that more detailed studies are needed to define the deficiencies.

At the 6 hr sampling, the PTT increased to 40.0 sec from a prechallenge mean of 27.4 sec. This abnormality remained during the first week after challenge except for a shortening to the upper normal range at 24 hrs post-challenge. After the first week no other prolongation was present. The PTT is commonly prolonged with a deficiency of any factor except VII (SPCA). This test in conjunction with the PT, would indicate that more than one clotting factor is deficient in these animals.

### Summary

Ten beagle dogs were given highly purified staphylococcal enterotoxin B intravenously and were observed and bled at intervals during a 55-day period. Pre- and postchallenge determinations of the whole blood clotting time, clot retraction, clot lysis, total white and differential cell count, prothrombin time, and partial thromboplastin time were performed on each sample. An abnormality was indicated by each test at some point during the testing period with the exception of clot lysis. The most frequent and more severe abnormalities occurred within the first four days after challenge. The results indicate that

alterations of the clotting mechanism do occur after administration of staphylococcal enterotoxin and suggests that the plasma factors are primarily involved. Changes in the leukocytic response appear to be similar to those sometimes observed in a severe bacterial infection.

### Résumé

On a administré par voie intraveineuse à 10 chiens courants de l'entérotoxine B hautement purifiée à partir de staphylocoques. Les animaux ont été observés et saignés à divers intervalles pendant une période de 55 jours. On a déterminé avant et après injection le temps de coagulation, la rétraction du caillot, la lyse du caillot, le nombre des globules blancs et leur distribution, le temps de prothrombine et le temps de thromboplastine partiel sur chaque échantillon. La lyse du caillot mise à part, on a observé des valeurs anormales pour chacun des tests à un moment ou l'autre de la période de contrôle. Les anomalies les plus fréquentes et les plus graves ont été observées pendant les 4 premiers jours. Les résultats indiquent des altérations du mécanisme de coagulation après administration de l'entérotoxine de staphylocoques et suggèrent que les facteurs plasmatiques sont atteints en premier lieu. Les altérations des leucocytes semblent être similaires à celles qu'on observe parfois lors d'une infection bactérienne grave.

### Zusammenfassung

10 Spürhunde erhielten hochgereinigtes Staphylokokken Enterotoxin B intravenös und wurden über 55 Tage hin beobachtet. In Abständen wurde das Blut untersucht. Vor und nach der Injektion wurden die Vollblutgerinnungszeit, die Retraktion, die Lyse, die Gesamtzahl der weißen Blutkörperchen und das Differentiaibild, die Prothrombinzeit und die partielle Thromboplastinzeit bestimmt. Ein abnormes Ergebnis wurde mit jedem Test zu irgendeinem Zeitpunkt während der Beobachtungsperiode beobachtet, nur die Lyse blieb unverändert. Die häufigsten und schwersten Veränderungen wurden in den ersten 4 Tagen nach der Injektion beobachtet. Die Ergebnisse zeigen, daß Veränderungen des Gerinnungsmechanismus nach der Gabe von Staphylokokken-Enterotoxin auftreten, und daß vorwiegend die Plasmafaktoren betroffen sind. Die Veränderungen im Verhalten der Leukozyten scheinen jenen bei schweren Infektionen ähnlich zu sein.

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